Gene expression profiling of human HBV- and/or HCV-associated hepatocellular carcinoma cells using expressed sequence tags

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Abstract. Liver cancer is one of the leading causes of cancer death worldwide. To identify novel target genes that are related to liver carcinogenesis, we examined new genes that are differentially expressed in human hepatocellular carcinoma (HCC) cell lines and tissues based on the expressed sequence tag (EST) frequency. Eleven libraries were constructed from seven HCC cell lines and three normal liver tissue samples obtained from Korean patients. An analysis of gene expression profiles for HCC was performed using the frequency of ESTs obtained from these cDNA libraries. Genes were identified (n=120) as being either up- or down-regulated in human liver cancer cells. Among these, 14 genes (*FTL*, *K-ALPHA1*, *LDHA*, *RPL4*, *ENO1*, *ANXA2*, *RPL9*, *RPL10*, *RPL13A*, *GNB2L1*, *AMBP*, *GC*, *A1BG*, and *SERPINC1*), in addition to previously well-known liver cancer related genes, were confirmed to be differentially expressed in seven liver cancer cell lines and 17 HCC tissues by semi-quantitative RT-PCR. In addition, 73 genes, in which there was a significant difference (P>0.99) between HBV- and HCV-associated HCC cells, were selected. Of these, expression patterns of 14 (*RPLP0*, *AKR1C*, *KRT8*, *GPX4*, *RPS15*, *ID1*, *RPS21*, *VIM*, *EEF1G*, *EIF4A1*, *HLA-C*, *FN1*, *CD44*, and *RPS10*) were confirmed by semi-quantitative RT-PCR in four of HBV- and three of HCV-associated HCC cell lines. Among those genes, an immuno-histochemical analysis for ANXA2 showed that it is expressed at high levels in HCC. Using an analysis of EST frequency, the newly identified genes, especially ANXA2, represent potential biomarkers for HCC and useful targets for elucidating the molecular mechanisms associated with HCC involving virological etiology.

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Introduction

Liver cancer is one of the leading causes of cancer death worldwide. Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver. Major risk factors for HCC are chronic hepatitis resulting from infection with HBV or HCV and exposure to various exogenous carcinogens, including aflatoxin B1 (1). Studies have shown that the incidence of HCC has substantially increased in East Asia, including Korea and Japan (2,3). Since most patients suffering from HCC do not survive long-term, a great deal of interest and urgency has developed regarding identifying novel HCC diagnostic markers for early detection and cancer-specific related genes as potential therapeutic targets in the treatment of HCC (4). Therefore, different markers are needed to correctly diagnose HCC.

Liver carcinogenesis is a multi-step process involving a variety of genetic alterations. The molecular mechanisms of hepatocarcinogenesis are not well understood, although aneuploidy and multiple genetic alterations are frequently present. It is noteworthy that mutations of p53, Rb and ß-catenin, as well as the overexpression of c-myc and cyclin D1, have been reported in HCC (5-8). A loss of heterozygosity (LOH) at multichromosomal loci has also been frequently reported in human HCC (9-11). In addition, several growth factors such as TGF α and ß are known to be implicated in the development of HCC (12,13). An analysis of the gene expression profile in HCC using a microarray has also been reported (14-17). Shirota *et al* reported that transcriptional factors or tissue-specific expression proteins were related to cell differentiation or the development of HCC (14). In addition, it has been reported that mitosis-promoting genes are up-regulated in HCC (17). Genes involved in cell cycle regulation such as cyclin, cyclin-dependent kinases, and cell

Table I. Primer sequences for semi-quantitative RT-PCR.

Genes	Forward (5'→3')	Reverse (5'→3')
HCC vs. Normal		
FTL	ATGAGCTCCCAGATTCGTCA	CCAGGAAGTCACAGAGATGG
K-ALPHA-1	GGCTTCAAGGTTGGCATC	CCTCTCCTTCTTCCTCACC
LDHA	ATGGCAACTCTAAAGGATCA	GCAACTTGCAGTTCGGGC
RPL4	GAGCTGGCAAAGGCAAA	TCCGGCGCATGGTCTTT
EN01	GACTTGGCTGGCAACTCTG	GGTCATCGGGAGACTTGAA
ANXA2	ATGTCTACTGTTCACGAAAT	GCTCCTGGTTGGTTCTGG
RPL9	ATGAAGACTATTCTCAGCAATC	TGAACAAGCAACACCTGGTC
RPL10	ATGGGCCGCCGCCCCGCC	TGAGTGCAGGGCCCGCCA
RPL13A	ATGGCGGAGGTGCAGGTC	GACCAGGAGTCCGTGGGT
GNB2L1	AACCACATTGGCCACACA	TGCCAATGGTCACCTGC
AMBP	AGTGGTACAACCTGGCCATC	ACAGCCCTCCGGACTCTC
GC	ATGAAGAGGGTCCTGGTACTAC	TCATTTGTGGGTTCCACGTA
A1BG	ATGTCCATGCTCGTGGTCTT	TCAGGCACCTCCAGAAACTC
SERPINC1	ATGTATTCCAATGTGATAGGAA	TCAGTTGCTGGAGGGTGTC
HBV-HCC vs. HCV-HCC		
RPLPO	GTTGCTGGCCAATAAGGT	GCCAAGAAGGCCTTGACC
AKR1CN	AACTTCAACCACAGGCTGCT	ACACCTGCACGTTCTGTCTG
KRT8	CTGCTGGAGGGCGAGGA	CAGCGCAGGAGGGGTAG
GPX4	CAGTGAGGCAAGACCGAAGT	GGGGCAGGTCCTTCTCTATC
RPS15	CTTCCGCAAGTTCACCTACC	GGGCTTGTAGGTGATGGAGA
ID1	GGTGCGCTGTCTGTCTGAG	CTGATCTCGCCGTTGAGG
RPS21	GCGAGTTCGTGGACCTGTA	CCAATCGGAGAATGGAATCA
VIM	ATGTCCACCAGGTCCGTG	TCGTTGGTTAGCTGGTCCAC
EEF1G	AGGCGCTGGCTGCTGAG	CGTAGTCCACCTGCCAATC
EIF4A1	CTGGCCGTGTGTTTGATATG	TGTCCAGCTTCCACTCCTCT
HLA-C	TGGGGAGGACCAAACTCA	GCAGCTCCCTCCTTTTCC
FN1	CCAACCTACGGATGACTCGT	TGGCACCGAGATATTCCTTC
CD44	GGCCAGCAAGTCTCAGGA	AGGCCTCCAAGTGGGAAC
RPS10	AGACAAGAATGTGCCCAACC	GGGGCAGATGAAGGTAATCA
B2M	CTCGCTCCGTGGCCTTAG	CAAATGCGGCATCTTCAA

cycle negative regulators have also been reported to be down-regulated (15).

The application of global approaches such as the collection of expressed sequence tags (ESTs), Serial Analysis of Gene Expression (SAGE) and microarray techniques have been shown to be useful in the analysis of complex biological phenomena, including human diseases (15,17-21). Among these approaches, the expressed sequence tags (ESTs) generated by the single-pass sequencing of randomly selected cDNA clones from cDNA libraries have been used to identify novel genes (22). ESTs have also been found to be useful for the differential and quantitative analysis of expression patterns (23), and evaluation of gene expression profiles in a specific tissue (22-24). Therefore, it is evident that the identification and cataloging of genes associated with liver carcinogenesis, obtained by detailed gene expression profiles, would facilitate the elucidation of the molecular mechanisms of liver carcinogenesis.

To identify the genes associated with HCC by examining their expression profiles, we collected a large number of genes that are expressed in HCC cell lines or tissues. In particular, we applied a strategy for obtaining full-length cDNAs, since these clones are a valuable resource for the functional study of genes. As a first step, we constructed eleven full-length enriched cDNA libraries from HCC cell lines and tissues from Korean patients (25,26). Using the EST frequency obtained from the liver EST data, the expression profile of the genes expressed in HCC cell lines and tissues was analyzed, and genes that are differentially expressed between HCC and normal tissues were selected as candidate markers for the diagnosis of HCC. Here, we report on the identification of genes containing HBV and/or HCV that are related to HCC .

Materials and methods

Cell culture, tissue samples and RNA preparation. The four different cell lines established from Korean HCC patients, SNU354, SNU368, SNU387 and SNU475, were hepatitis B surface antigen-positive and obtained from the Korean Cell Line Bank (http://cellbank.snu.ac.kr/) (27). Three different cell lines established from Korean HCC patients with HCV-positive HLK1, HLK3 and JSHC were obtained from the Chonbuk National University Medical School and Hospital, Korea (28). All seven cell lines were maintained at 37˚C in humidified air containing 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum.

The three normal liver tissues, N779227, N800102 and N803806, were used to construct the cDNA libraries, and obtained from necropsy in the Catholic University of Medicine, Daejeon, Korea. Seventeen tissues with pairs of HCC tumors and adjacent non-tumor tissue were collected at the Catholic University of Medicine, Seoul, Korea. All patients participating in this study gave their informed consent before surgery. The HCCs were classified as grade I (n=3), II (n=5), III (n=5) and IV (n=4), respectively, corresponding to the criteria of Edmondson and Steiner (29).

Total RNA was extracted from the cultured cells and tissues using a commercially available RNA Isolation kit (Qiagen, Hilden, Germany) following the procedures recommended by the manufacturer.

Construction of cDNA library and DNA sequencing. The full-length cDNA library was constructed using an improved capping method (25). Plasmid DNAs were extracted from clones that had been randomly selected from the constructed cDNA libraries using an MWG Plasmidprep 96 (MWG Biotechnology, Ebersberg, Germany). DNA sequencing was performed using previously described procedures (30).

Bioinformatic analysis of ESTs. Analysis of the collected ESTs with a bioinformatic tool was performed following previously described procedures (30). The annotation of 'high quality' liver ESTs were carried out using the human mRNA subset extracted from the GenBank database and UniGene database (Hs.seq.all, build #163) for similarity comparisons using BLASTN. For a protein similarity assessment, a comparison was performed against the non-redundant protein database using BLASTX.

Gene expression analysis. The frequency of each gene was analyzed by dividing the number of ESTs of a gene by the number of total clones merged into the UniGene database build #163 in each library. Genes that were abundantly expressed in each cDNA library were selected and listed from among ESTs showing an expression frequency of ≥0.11%. Significant differences in gene expression between the data sets were calculated using a previously described method (31). Analysis of expressional differences between the normal pool and HCC pool was performed with a cut-off probability of 0.999. The same analysis, substituting an expression frequency of ≥0.1% and a cut-off probability of 0.99, was also performed for the HBV pool and HCV pool. The gene list was sorted according to gene frequency in the pool of the overexpressing gene.

The Gene Ontology (GO) database (www.geneontology.org/#godatabase) was used to classify the gene profiles into the gene ontology. Each UniGene cluster was mapped to LocusLink (as of April 21, 2004), then assigned GO IDs, as in the LocusLink database.

Semi-quantitative RT-PCR. Reverse transcription (RT) was performed with 5 μg of isolated RNA using previously described procedures (30). To quantify the amounts of first cDNAs, the RT solution mixed with a human B2M competitor DNA was used as the template for PCR. PCR was performed according to the method of Kim *et al* (30); conditions were 1 cycle for 2 min at 94˚C, and 25 cycles of 94˚C for 30 sec, 55˚C for 1 min, and 72˚C for 1 min with B2M primers (Table I). The concentration of the first cDNA of each sample was adjusted based on the amounts of PCR product of B2M competitor DNA. To validate the expression level of the selected genes, PCR was performed using adjusted first cDNAs as templates and a specific primer set for each gene (Table I) with the same conditions used in the above PCR. The transcriptional activity of each gene in the HCC cell lines was calculated relative to the average amount of target gene in normal tissues for up-regulated genes in the HCC pool or relative to that of cancer cells for down-regulated genes in the HCC pool, and are presented as the relative fold expression change (log base 2) after normalization against a B2M standard. On the other hand, the transcript levels of target genes in HCC tissues were calculated relative to the amount of target gene in the corresponding non-tumor liver tissue, and are presented as the relative fold expression change (log base 2) after normalization against B2M. In addition, the transcript levels of the up- or down-regulated target genes in HBV-HCC cell lines were calculated using the same method, except that they were done relative to the average amount of target gene in the HCV pool for up-regulation in the HBV pool or relative to that of the HBV pool for up-regulation in the HCV pool. The amount of B2M product in each cell line and tissue was calculated from a standard curve.

Immunohistochemistry. Immunohistochemical staining of ANXA2 in HCC tissues was performed using the same previously described procedures (32). A goat polyclonal antibody against ANXA2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as the primary antibody. These antibodies were used at a dilution of 1:100 for ANXA2.

Results

Large-scale ESTs collection from human HCC cells and tissues. Eleven full-length cDNA libraries were constructed from seven HCC cell lines and three normal liver tissues obtained from Korean patients. A total of 20,354 clones were randomly selected from these 10 libraries and used for 5' end single-pass sequencing. The obtained sequences were subjected to quality control procedures, namely trimming of the vector region and removal of low quality or short (<100 bp) sequences. Finally, 18,831 high quality ESTs with an average length of 500 bp were collected (Table II). After screening

Table II. Summary of cDNA libraries.

Source	Library	Vector	Reads	Unigene 163[a]	
				Clones	Clusters
HCC cell lines					
HBV-HCC					
SNU475	L3 SNU475	pCNS-D2	2336	2233	1011
SNU368	L4SNU368	pCNS-D2	2524	2445	834
SNU354	L9SNU354	pT7T3-Pac	1516	1194	913
	L11SNU354	pCNS-D2	1958	1627	1089
SNU387	L13SNU387	pCNS-D2	1181	1153	545
HCV-HCC					
HLK1	L5HLK1	pCNS-D2	3601	3495	1492
JSHC	L12JSHC	pCNS-D2	1005	981	556
HLK3	L16HLK3	pT7T3-Pac	2429	2110	1426
Normal tissues					
N800102	L7N800102	pCNS-D2	1466	1349	503
N779227	L19N779227	pCNS-D2	1254	1209	373
N803806	L20N803806	pCNS-D2	1084	1035	331
Total			20354	18831	5901

[a]Number of clones and clusters in NCBI Unigene Build 163 contributed by our EST sequences.

out the ESTs derived from mitochondrial DNAs, ribosomal DNAs and human repetitive sequences, the remaining sequences were submitted to the NCBI dbEST database (accession nos. CB105131-CB164813). When all of our ESTs were annotated by coalescing into human UniGene clusters (build 163), they were assembled into 5,901 clusters. Most were ESTs coding a known gene having an identity of at least 95% with human Refseq or mRNA. These annotated results were used in subsequent analyses of expression profiles.

Identification of HCC related genes. To identify the candidate genes related to human liver carcinogenesis, we divided the constructed libraries into two groups, a 'normal pool' and an 'HCC pool'. The normal pool was composed of three libraries of normal tissues and the HCC pool of eight libraries of cancer cells. We then selected 120 genes that had a frequency of >0.11% on average in each pool and showed a significant difference (P>0.999) between the two pools. We found 40 up-regulated genes and 80 down-regulated genes in the HCC pool, as shown in Table III.

Among the up-regulated genes in the HCC pool, significant differences were observed in the gene groups associated with cell structure formation (*K-ALPHA-1* and *ACTB*), the glycolysis pathway (*LDHA*, *ENO1*, and *PGK1*), heat shock proteins (*HSPA8* and *HSPCB*), cytoskeletal development (*ANXA2* and *TMSB10*), and protein synthesis (*RPL4*, *RPL9*, *RPLP0*, *RPL10*, *RPL13A*, *RPS8*, *RPL5*, *RPS12*, *RPL13*, *RPS20*, *RPL18A*, *RPS3A*, *RPS3*, and *RPS10*). Although gene *EEF1A1* was relatively abundant in the normal pool, the frequency of this gene in the HCC pool was significantly higher than in the normal pool.

In the case of down-regulated genes in the HCC pool, significant differences were observed in the gene groups related to liver-specific genes (*ALB*, *ORM1*, *C1R*, and *C3*), proteolysis and peptidolysis (*HP*, *HPR*, *CPB2*, and *ACY1*), regulation and blood pressure (*FGG*, *FGB*, *KNG*, and *AGT*), detoxification and drug metabolism (*CYP2A6*, *MT2A*, *CYP2A7*, *CYP2E1*, and *CYP2C8*), lipid metabolism (*APOH*, *APOA5*, *RODH-4*, *CYP3A4*, and *APOA1*), glycometabolism (*HPD* and *FBP1*), alcohol metabolism (*ADH1C*, *ADH1B*, and *ADH1A*), amino acid metabolism (*AGXT* and *TAT*), and the immune system (*CD74*, *CD14*, *AZGP1*, and *SLC22A1*).

Verification of HCC-related genes using semi-quantitative RT-PCR. To validate the up- and down-regulated genes in HCC selected from the EST frequency data, we randomly selected 13 up-regulated genes from the HCC pool (*FTL*, *K-ALPHA-1*, *LDHA*, *RPL4*, *ACTB*, *ENO1*, *ANXA2*, *RPL9*, *RPLP0*, *RPL10*, *VIM*, *RPL13A*, and *GNB2L1*), and 10 commonly down-regulated genes from the HCC pool (*ALB*, *HP, AMBP*, *FGG*, *GC*, *FGB*, *A1BG*, *VTN*, *SERPINC1*, and *FGA*), and performed semi-quantitative RT-PCR on HCC cell lines and tumor tissues.

Fig. 1 reveals the expression levels of the 10 up-regulated genes and four down-regulated genes in liver cancers, except for the previously reported liver cancer related genes such as *RPLP0*, *ACTB*, *VIM*, *ALB*, *HP*, *FGG*, *FGB*, *VTN* and *FGA*. As shown in Fig. 1A, the results of RT-PCR in HCC cell lines show that all of the up-regulated genes were highly expressed in most of the HCC cell lines, but were either expressed at very low levels or not at all in normal tissues. On the other hand, all down-regulated genes were expressed at very low levels or not detected in all HCC cell lines, and

Table III. Genes showing a significant difference in expression (p>0.999[a]) between normal liver tissues and liver cancer cell lines.

UniGene	Copies in normal (%)	Copies in HCC (%)	Gene	Chromosome	Title	GO Id	GO Term
Up-regulated in cancer cell							
Hs.439552	63 (1.75)	< 620 (4.07)	EEF1A1	6q14.1	eukaryotic translation elongation factor 1 alpha 1	GO:0006414	translational elongation
Hs.433670	6 (0.17)	< 299 (1.96)	FTL	19q13.3-q13.4	ferritin, light polypeptide	GO:0006879	iron ion homeostasis
Hs.446608	6 (0.17)	< 258 (1.69)	K-ALPHA-1	12q13.12	tubulin, alpha, ubiquitous	GO:0051258	protein polymerization
Hs.2795	6 (0.17)	< 195 (1.28)	LDHA	11p15.4	lactate dehydrogenase A	GO:0006096	glycolysis
Hs.186350	12 (0.33)	< 124 (0.81)	RPL4	15q22	ribosomal protein L4	GO:0006412	protein biosynthesis
Hs.426930	3 (0.08)	< 123 (0.81)	ACTB	7p15-p12	actin, beta	GO:0006928	cell motility
Hs.433455	5 (0.14)	< 111 (0.73)	ENO1	1p36.3-p36.2	enolase 1, (alpha)	GO:0000122	negative regulation of transcription from Pol II
Hs.462864	2 (0.06)	< 103 (0.68)	ANXA2	15q21-q22	annexin A2	GO:0001501	skeletal development
Hs.412370	2 (0.06)	< 97 (0.64)	RPL9	4p13	ribosomal protein L9	GO:0006412	protein biosynthesis
Hs.443796	0 (0.00)	< 94 (0.62)	RPLP0	12q24.2	ribosomal protein, large, P0	GO:0006414	translational elongation
Hs.401929	1 (0.03)	< 92 (0.60)	RPL10	Xq28	ribosomal protein L10	GO:0006412	protein biosynthesis
Hs.435800	2 (0.06)	< 84 (0.55)	VIM	10p13	vimentin		
Hs.449070	0 (0.00)	< 72 (0.47)	RPL13A	19q13.3	ribosomal protein L13a	GO:0006412	protein biosynthesis
Hs.512675	0 (0.00)	< 67 (0.44)	RPS8	1p34.1-p32	ribosomal protein S8	GO:0006412	protein biosynthesis
Hs.469653	0 (0.00)	< 67 (0.44)	RPL5	1p22.1	ribosomal protein L5	GO:0006412	protein biosynthesis
Hs.295131	2 (0.06)	< 67 (0.44)	AKR1C1	10p15-p14	aldo-keto reductase family 1, member C1	GO:0006805	xenobiotic metabolism
Hs.5662	0 (0.00)	< 66 (0.43)	GNB2L1	5q35.3	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	GO:0007205	protein kinase C activation
Hs.76392	1 (0.03)	< 62 (0.41)	ALDH1A1	9q21.13	aldehyde dehydrogenase 1 family, member A1	GO:0006081	aldehyde metabolism
Hs.380956	0 (0.00)	< 59 (0.39)	RPS12	6q23.1	ribosomal protein S12	GO:0006412	protein biosynthesis
Hs.410817	1 (0.03)	< 56 (0.37)	RPL13	16q24.3	ribosomal protein L13	GO:0006412	protein biosynthesis
Hs.8102	0 (0.00)	< 53 (0.35)	RPS20	8q12	ribosomal protein S20	GO:0006412	protein biosynthesis
Hs.356331	1 (0.03)	< 48 (0.32)	PPIA	7p13-p11.2	peptidylprolyl isomerase A (cyclophilin A)	GO:0006457	protein folding
Hs.337766	0 (0.00)	< 46 (0.30)	RPL18A	19p13	ribosomal protein L18a	GO:0006412	protein biosynthesis
Hs.374596	1 (0.03)	< 44 (0.29)	TPT1	13q12-q14	tumor protein, translationally-controlled 1		
Hs.356572	0 (0.00)	< 44 (0.29)	RPS3A	4q31.2-q31.3	ribosomal protein S3A	GO:0006412	protein biosynthesis
Hs.374553	1 (0.03)	< 43 (0.28)	LAMR1	3p21.3	laminin receptor 1 (ribosomal protein SA, 67kDa)	GO:0007155	cell adhesion
Hs.50273	0 (0.00)	< 41 (0.27)	DKFZP434I1	8q22.1	DKFZP434I116 protein		
Hs.513041	0 (0.00)	< 40 (0.26)	C15orf12	15q24	chromosome 15 open reading frame 12	GO:0006364	rRNA processing
Hs.78771	0 (0.00)	< 39 (0.26)	PGK1	Xq13	phosphoglycerate kinase 1	GO:0006096	glycolysis
Hs.180414	0 (0.00)	< 39 (0.26)	HSPA8	11q24.1	heat shock 70kDa protein 8	GO:0006412	protein biosynthesis
Hs.419776	0 (0.00)	< 37 (0.24)	NAP1L1	12q21.1	nucleosome assembly protein 1-like 1	GO:0006260	DNA replication
Hs.489336	0 (0.00)	< 36 (0.24)	SYAP1	Xp22.22	synapse associated protein 1, SAP47 homolog (Drosophila)		
Hs.5120	0 (0.00)	< 35 (0.23)	DNCL1	12q24.23	dynein, cytoplasmic, light polypeptide 1	GO:0007017	microtubule-based process
Hs.301404	0 (0.00)	< 35 (0.23)	RBM3	Xp11.2	RNA binding motif protein 3	GO:0003723	RNA binding
Hs.438678	0 (0.00)	< 34 (0.22)	TALDO1	11p15.5-p15.4	transaldolase 1	GO:0005975	carbohydrate metabolism
Hs.387576	0 (0.00)	< 34 (0.22)	RPS3	11q13.3-q13.5	ribosomal protein S3	GO:0006412	protein biosynthesis
Hs.446574	0 (0.00)	< 34 (0.22)	TMSB10	2p11.2	thymosin, beta 10	GO:0007010	cytoskeleton organization and biogenesis
Hs.406620	0 (0.00)	< 33 (0.22)	RPS10	6p21.31	ribosomal protein S10	GO:0006412	protein biosynthesis
Hs.459927	0 (0.00)	< 32 (0.21)	PTMA	2q35-q36	prothymosin, alpha (gene sequence 28)	GO:0006350	transcription
Hs.74335	1 (0.03)	< 38 (0.25)	HSPCB	6p12	heat shock 90kDa protein 1, beta	GO:0006986	response to unfolded protein
Down-regulated in cancer cell							
Hs.418167	530 (14.75)	> 0 (0.00)	ALB	4q11-q13	albumin		
Hs.403931	379 (10.55)	> 0 (0.00)	HP	16q22.1	haptoglobin	GO:0006508	proteolysis and peptidolysis
Hs.76177	87 (2.42)	> 1 (0.01)	AMBP	9q32-q33	alpha-1-microglobulin/bikunin precursor	GO:0006810	transport
Hs.75431	74 (2.06)	> 0 (0.00)	FGG	4q28	fibrinogen, gamma polypeptide	GO:0008217	regulation of blood pressure
Hs.418497	66 (1.84)	> 0 (0.00)	GC	4q12-q13	group-specific component (vitamin D binding protein)	GO:0015875	vitamin/cofactor transport
Hs.300774	64 (1.78)	> 1 (0.01)	FGB	4q28	fibrinogen, B beta polypeptide	GO:0008217	regulation of blood pressure
Hs.525562	61 (1.70)	> 1 (0.01)	-	14	similar to protein sp:P01011 AACT_HUMAN Alpha-1-antichymotrypsin precursor (ACT)		
Hs.390608	58 (1.61)	> 0 (0.00)	A1BG	19q13.4	alpha-1-B glycoprotein	GO:0000004	biological process unknown
Hs.2257	58 (1.61)	> 1 (0.01)	VTN	17q11	vitronectin (serum spreading factor, somatomedin B, complement S-protein)	GO:0007155	cell adhesion
Hs.75599	57 (1.59)	> 0 (0.00)	SERPINC1	1q23-q25.1	serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1		
Hs.351593	55 (1.53)	> 0 (0.00)	FGA	4q28	fibrinogen, A alpha polypeptide		
Hs.512155	50 (1.39)	> 0 (0.00)	HPR	16q22.1	haptoglobin-related protein	GO:0006508	proteolysis and peptidolysis
Hs.2523	44 (1.22)	> 2 (0.01)	ADH1C	4q21-q23	alcohol dehydrogenase 1C (class I), gamma polypeptide	GO:0006066	alcohol metabolism
Hs.446471	44 (1.22)	> 2 (0.01)	CD74	5q32	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	GO:0006955	immune response
Hs.297681	40 (1.11)	> 1 (0.01)	SERPINA1	14q32.1	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	GO:0006953	acute-phase response
Hs.2899	40 (1.11)	> 1 (0.01)	HPD	12q24-qter	4-hydroxyphenylpyruvate dioxygenase	GO:0006572	tyrosine catabolism
Hs.324746	34 (0.95)	> 0 (0.00)	AHSG	3q27	alpha-2-HS-glycoprotein	GO:0001503	ossification
Hs.77741	33 (0.92)	> 0 (0.00)	KNG	3q27	kininogen	GO:0008217	regulation of blood pressure
Hs.439056	30 (0.83)	> 0 (0.00)	CYP2A6	19q13.2	cytochrome P450, family 2, subfamily A, polypeptide 6	GO:0006118	electron transport
Hs.426485	26 (0.72)	> 0 (0.00)	HPX	11p15.5-p15.4	hemopexin	GO:0015886	heme transport
Hs.4	24 (0.67)	> 0 (0.00)	ADH1B	4q21-q23	alcohol dehydrogenase IB (class I), beta polypeptide	GO:0006069	ethanol oxidation
Hs.19383	20 (0.56)	> 1 (0.01)	AGT	1q42-q43	angiotensinogen (serine (or cysteine) proteinase inhibitor, clade A	GO:0008217	regulation of blood pressure
Hs.422655	20 (0.56)	> 0 (0.00)	ADH1A	4q21-q23	alcohol dehydrogenase 1A (class I), alpha polypeptide	GO:0006066	alcohol metabolism
Hs.75627	18 (0.50)	> 0 (0.00)	CD14	5q22-q32	CD14 antigen	GO:0006909	phagocytosis
Hs.76530	16 (0.45)	> 0 (0.00)	F2	11p11-q12	coagulation factor II (thrombin)	GO:0007260	tyrosine phosphorylation of STAT protein
Hs.333497	15 (0.42)	> 0 (0.00)	CYP2D6	22q13.1	cytochrome P450, family 2, subfamily D, polypeptide 6		
Hs.1252	15 (0.42)	> 0 (0.00)	APOH	17q23-qter	apolipoprotein H (beta-2-glycoprotein I)		
Hs.497571	13 (0.36)	> 0 (0.00)	-	14	similar to protein sp:P01011 AACT_HUMAN Alpha-1-antichymotrypsin precursor (ACT)		
Hs.283923	13 (0.36)	> 0 (0.00)	APOA5	11q23	apolipoprotein A-V	GO:0006869	lipid transport
Hs.384598	13 (0.36)	> 0 (0.00)	SERPING1	11q12-q13.1	serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)		
Hs.418241	12 (0.33)	> 1 (0.01)	MT2A	16q13	metallothionein 2A	GO:0006823	heavy metal ion transport
Hs.278388	12 (0.33)	> 1 (0.01)	ORM1	9q31-q32	orosomucoid 1	GO:0006810	transport
Hs.308680	11 (0.31)	> 0 (0.00)	C1R	12p13	complement component 1, r subcomponent	GO:0006958	compelement activation, classical pathway
Hs.1305	10 (0.28)	> 0 (0.00)	SERPINA6	14q32.1	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6	GO:0006810	transport
Hs.107	10 (0.28)	> 0 (0.00)	FGL1	8p22-p21.3	fibrinogen-like 1		
Hs.2351	9 (0.25)	> 1 (0.01)	PROC	2q13-q14	protein C (inactivator of coagulation factors Va and VIIIa)		
Hs.134958	9 (0.25)	> 0 (0.00)	RODH-4	12q13.13-q13.2	microsomal NAD+-dependent retinol dehydrogenase 4	GO:0006629	lipid metabolism
Hs.458355	9 (0.25)	> 5 (0.03)	C1S	12p13	complement component 1, s subcomponent	GO:0006958	compelement activation, classical pathway
Hs.69771	8 (0.22)	> 2 (0.01)	BF	6p21.3	B-factor, properdin	GO:0006508	proteolysis and peptidolysis
Hs.221926	8 (0.22)	> 0 (0.00)	CPB2	13q14.11	carboxypeptidase B2 (plasma, carboxypeptidase U)	GO:0006508	proteolysis and peptidolysis
Hs.442527	8 (0.22)	> 0 (0.00)	CYP3A4	7q21.1	cytochrome P450, family 3, subfamily A, polypeptide 4	GO:0006629	lipid metabolism
Hs.420257	8 (0.22)	> 0 (0.00)	ITIH1	3p21.2-p21.1	inter-alpha (globulin) inhibitor, H1 polypeptide	GO:0006953	acute-phase response
Hs.282209	7 (0.19)	> 0 (0.00)	PON1	7q21.3	paraoxonase 1	GO:0009605	response to external stimulus
Hs.144567	7 (0.19)	> 0 (0.00)	AGXT	2q36-q37	alanine-glyoxylate aminotransferase (oxalosis I; hyperoxaluria I; glycolicaciduria)		
Hs.1498	7 (0.19)	> 0 (0.00)	HRG	3q27	histidine-rich glycoprotein		
Hs.250615	7 (0.19)	> 0 (0.00)	CYP2A7	19q13.2	cytochrome P450, family 2, subfamily A, polypeptide 7	GO:0006118	electron transport
Hs.156316	7 (0.19)	> 1 (0.01)	DCN	12q13.2	decorin	GO:0009887	organogenesis
Hs.334707	7 (0.19)	> 0 (0.00)	ACY1	3p21.1	aminoacylase 1	GO:0006508	proteolysis and peptidolysis
Hs.74561	7 (0.19)	> 1 (0.01)	A2M	12p13.3-p12.3	alpha-2-macroglobulin	GO:0006886	intracellular protein transport
Hs.76415	7 (0.19)	> 0 (0.00)	ITIH4	3p21-p14	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)		
Hs.512643	7 (0.19)	> 1 (0.01)	AZGP1	7q22.1	alpha-2-glycoprotein 1, zinc	GO:0006955	immune response
Hs.117367	7 (0.19)	> 0 (0.00)	SLC22A1	6q26	solute carrier family 22 (organic cation transporter), member 1		
Hs.11881	7 (0.19)	> 0 (0.00)	TM4SF4	3q25	transmembrane 4 superfamily member 4	GO:0008285	negative regulation of cell proliferation
Hs.433923	7 (0.19)	> 0 (0.00)	TF	3q21	transferrin		
Hs.1259	6 (0.17)	> 0 (0.00)	ASGR2	17p	asialoglycoprotein receptor 2	GO:0006897	endocytosis
Hs.284394	6 (0.17)	> 1 (0.01)	C3	19p13.3-p13.2	complement component 3	GO:0006958	compelement activation, classical pathway
Hs.75183	6 (0.17)	> 0 (0.00)	CYP2E1	10q24.3-qter	cytochrome P450, family 2, subfamily E, polypeptide 1	GO:0006118	electron transport
Hs.278568	6 (0.17)	> 1 (0.01)	HF1	1q32	H factor 1 (complement)	GO:0006957	compelement activation, alternative pathway
Hs.93194	6 (0.17)	> 0 (0.00)	APOA1	11q23-q24	apolipoprotein A-I	GO:0006869	lipid transport
Hs.150833	6 (0.17)	> 0 (0.00)	C4A	6p21.3	complement component 4A	GO:0006956	complement activation
Hs.333303	5 (0.14)	> 0 (0.00)	GJB1	Xq13.1	gap junction protein, beta 1, 32kDa (connexin 32, Charcot-Marie-Tooth neuropathy, X-linked)		
Hs.143436	5 (0.14)	> 0 (0.00)	PLG	6q26	plasminogen	GO:0008285	negative regulation of cell proliferation
Hs.296941	5 (0.14)	> 0 (0.00)	HFL1	1q32	H factor (complement)-like 1		
Hs.309583	5 (0.14)	> 1 (0.01)	SLC27A5	19q13.43	solute carrier family 27 (fatty acid transporter), member 5		
Hs.284712	5 (0.14)	> 0 (0.00)	BAAT	9q22.3	bile acid Coenzyme A: amino acid N-acyltransferase (glycine N-choloyltransferase)	GO:0006544	glycine metabolism
Hs.427202	5 (0.14)	> 0 (0.00)	TTR	18q12.1	transthyretin (prealbumin, amyloidosis type I)	GO:0006810	transport
Hs.292682	5 (0.14)	> 0 (0.00)	LOC348158	16p13.11	xenobiotic/medium-chain fatty acid:CoA ligase	GO:0008152	metabolism
Hs.93675	5 (0.14)	> 1 (0.01)	DEPP	10q11.21	decidual protein induced by progesterone		
Hs.271167	5 (0.14)	> 0 (0.00)	PIPOX	17q11.2	pipecolic acid oxidase	GO:0046653	tetrahydrofolate metabolism
Hs.282871	5 (0.14)	> 0 (0.00)	CYP2C8	10q24.1	cytochrome P450, family 2, subfamily C, polypeptide 8	GO:0006118	electron transport
Hs.38069	4 (0.11)	> 0 (0.00)	C8B	1p32	complement component 8, beta polypeptide	GO:0009618	response to pathogenic bacteria
Hs.193640	4 (0.11)	> 0 (0.00)	HAO1	20p12	hydroxyacid oxidase (glycolate oxidase) 1		
Hs.251664	4 (0.11)	> 0 (0.00)	na	11p15.5	similar to putative insulin-like growth factor ii associated protein		
Hs.236545	4 (0.11)	> 0 (0.00)	HAO2	1p13.3-p13.1	hydroxyacid oxidase 2 (long chain)		
Hs.32603	4 (0.11)	> 0 (0.00)	COLEC11	2p25.3	collectin sub-family member 11		
Hs.76452	4 (0.11)	> 0 (0.00)	CRP	1q21-q23	C-reactive protein, pentraxin-related	GO:0006953	acute-phase response
Hs.161640	4 (0.11)	> 0 (0.00)	TAT	16q22.1	tyrosine aminotransferase		
Hs.512587	4 (0.11)	> 0 (0.00)	MST1	3p21	macrophage stimulating 1 (hepatocyte growth factor-like)		
Hs.360509	4 (0.11)	> 0 (0.00)	FBP1	9q22.3	fructose-1,6-bisphosphatase 1	GO:0006000	fructose metabolism
Hs.81073	4 (0.11)	> 0 (0.00)	FETUB	3q27	fetuin B		

[a] Probability of differential expression calculated based on Audic and Claverie 1997. GO ids are based on process ontology of Gene Ontology database. Genes abundant (> 0.1%) in normal or cancer pool are listed. Colors indicate the frequency of a gene in each library.

0% 0. 0.5 1 ≥ 5

showed high expression levels in normal tissues (Fig. 1B). These results show that the transcriptional levels of the selected genes by semi-quantitative RT-PCR are consistent with the EST frequency data for all genes.

An expression analysis of the 14 confirmed genes in all 17 pairs of HCC tumor tissues and their corresponding non-tumor tissues in stages I-IV was also performed. The results showed that the up-regulated genes were highly expressed in

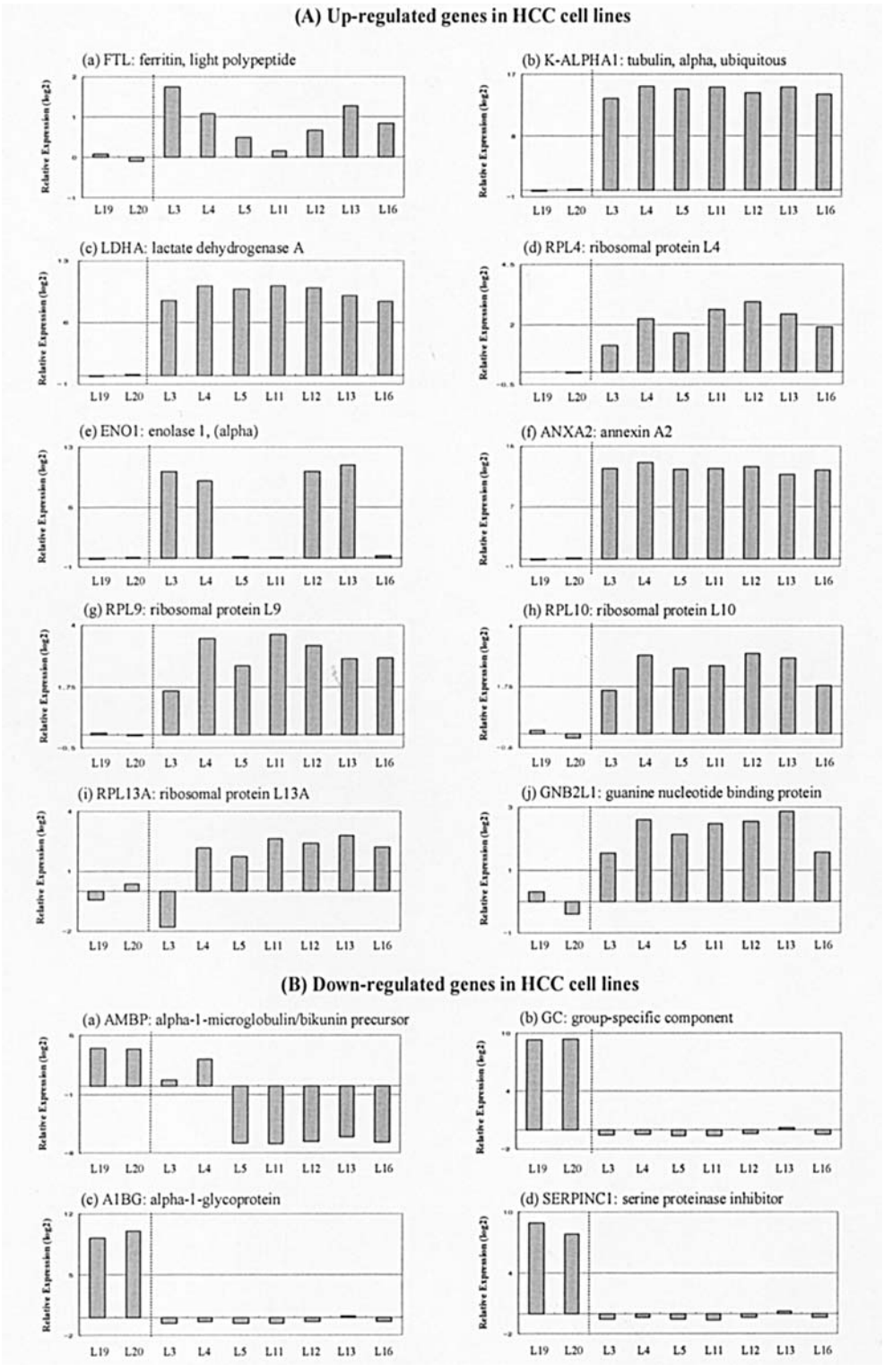


Figure 1. Semi-quantitative RT-PCR of the up- or down-regulated genes randomly selected from an HCC pool based on EST frequency in HCC cell lines. Total RNAs were extracted from normal tissues (L19, N779227; L20, N803806) and HCC cell lines (L3, SNU475; L4, SNU368; L5, HLK1; L11, SNU354; L12, JSHC; L13, SNU387; L16, HLK3) and used as templates for semi-quantitative RT-PCR (see Materials and methods for details). (A) Up-regulated and (B) down-regulated genes in the HCC pool. The transcript levels of target genes were calculated relative to the average amount of target gene in normal tissues (A) or relative to that of HCC cells (B), and are presented as the relative fold expression change (log base 2) after normalization against B2M. The amount of B2M product in each cell line and tissue was calculated from a standard curve. A positive value represents an up-regulated expression level, and a negative value represents a down-regulated expression level relative to the average amount of target gene.

most HCC tumor tissues compared to their corresponding non-tumor tissues (Fig. 2A). These genes were highly expressed in tumors with a frequency of 53%-77% in 17 HCC tissues. In particular, the highly expressed frequencies of these genes in HCC tumors were increased with increasing progression. In addition, the expression patterns of four down-regulated genes were also confirmed in HCC tumor tissues. These genes were expressed at low levels in HCC with a frequency of 59%-76% (Fig. 2B). The low expression level of these genes was detected in stages I to IV of HCC.

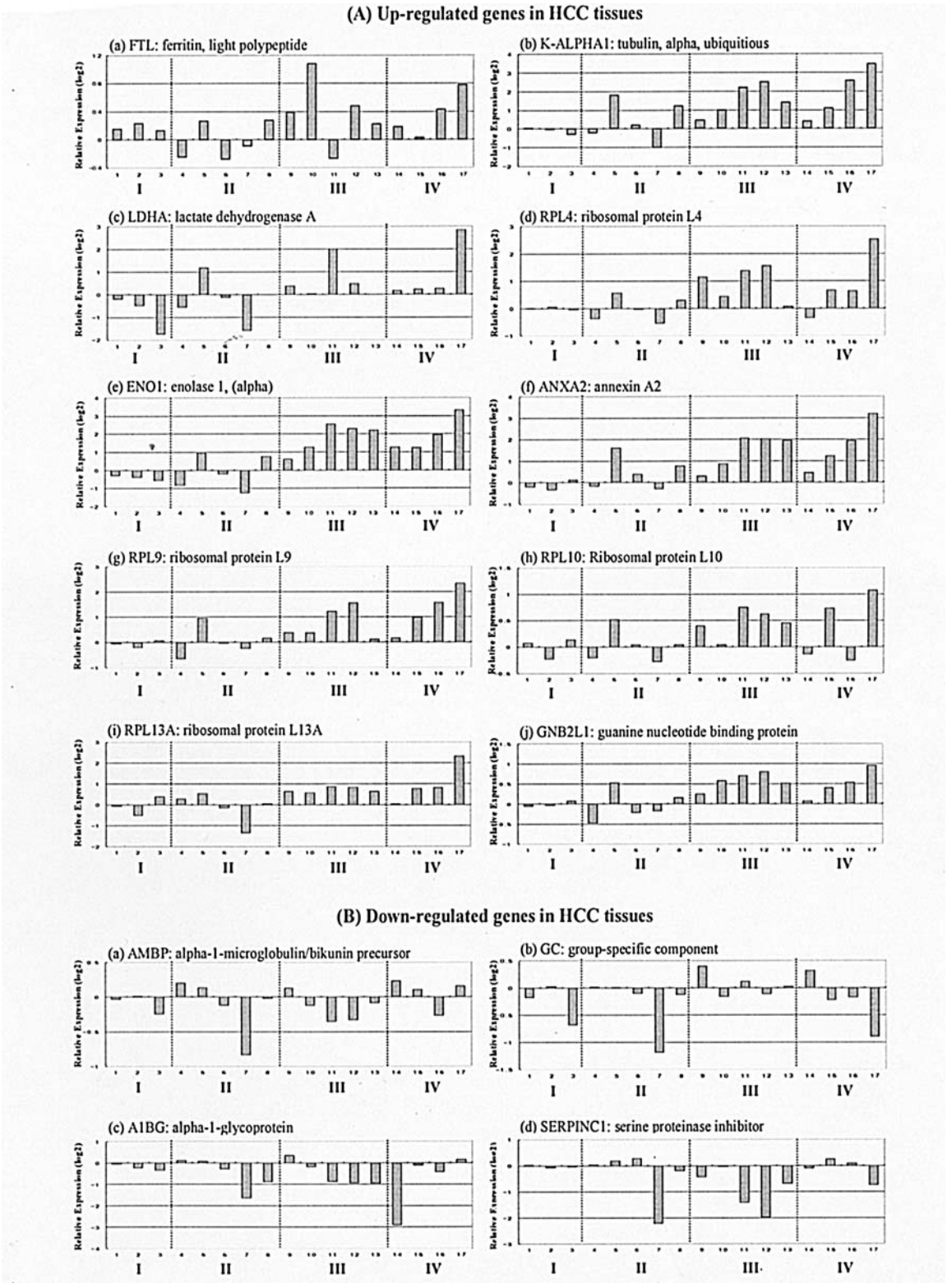
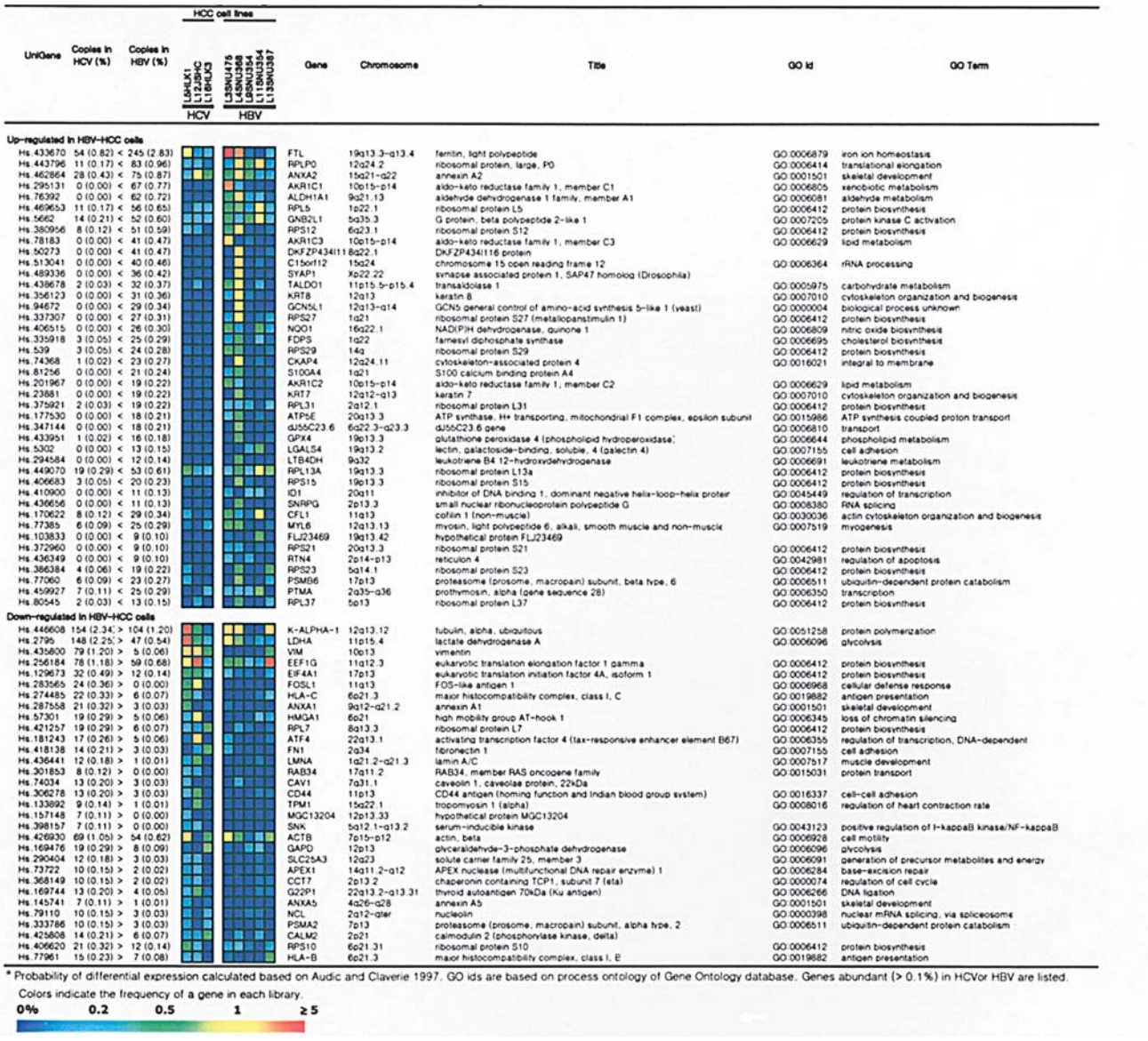


Figure 2. Semi-quantitative RT-PCR of the up- or down-regulated genes randomly selected from the HCC pool in HCC tissues. Total RNAs were extracted from 17 HCC tumor samples and their corresponding non-tumor liver tissue samples, then used as templates for semi-quantitative RT-PCR. (A) Up-regulated and (B) down-regulated genes in the HCC pool. The transcript levels of target genes were calculated relative to the amount of target gene in their corresponding non-tumor liver tissue, and are presented as the relative fold expression change (log base 2) after normalization against B2M. I, II, III, and IV designate classified HCC grades corresponding to Edmonson and Steiner.

These results indicate that the confirmed data from semi-quantitative RT-PCR are in good agreement with the EST frequency data. In addition, the mRNA levels of the target genes in liver tissues were largely consistent with those of the cell lines.

Identification of genes related to HBV- or HCV-associated HCC. It has clearly been established that HBV and HCV infections are major risk factors for the development of HCC (1). We further analyzed the EST frequencies between both libraries of the HBV- and HCV-associated HCC cell lines to

Table IV. Genes showing a significant difference in expression (p>0.99[a]) between HBV- and HCV-associated HCC cell lines.



UniGene	Copies in HCV (%)	Copies in HBV (%)	Gene	Chromosome	Title	GO id	GO Term
Up-regulated in HBV-HCC cells							
Hs.433670	54 (0.82) <	245 (2.83)	FTL	19q13.3-q13.4	ferritin, light polypeptide	GO:0006879	iron ion homeostasis
Hs.443796	11 (0.17) <	83 (0.96)	RPLP0	12q24.2	ribosomal protein, large, P0	GO:0006414	translational elongation
Hs.462864	28 (0.43) <	75 (0.87)	ANXA2	15q21-q22	annexin A2	GO:0001501	skeletal development
Hs.295131	0 (0.00) <	67 (0.77)	AKR1C1	10p15-p14	aldo-keto reductase family 1, member C1	GO:0006805	xenobiotic metabolism
Hs.76392	0 (0.00) <	62 (0.72)	ALDH1A1	9q21.13	aldehyde dehydrogenase 1 family, member A1	GO:0006081	aldehyde metabolism
Hs.46953	11 (0.17) <	56 (0.65)	RPL5	1p22.1	ribosomal protein L5	GO:0006412	protein biosynthesis
Hs.5662	14 (0.21) <	52 (0.60)	GNB2L1	5q35.3	G protein, beta polypeptide 2-like 1	GO:0007205	protein kinase C activation
Hs.380956	8 (0.12) <	51 (0.59)	RPS12	6q23.1	ribosomal protein S12	GO:0006412	protein biosynthesis
Hs.78183	0 (0.00) <	41 (0.47)	AKR1C3	10p15-p14	aldo-keto reductase family 1, member C3	GO:0006629	lipid metabolism
Hs.50273	0 (0.00) <	41 (0.47)	DKFZP434I116	8q22.1	DKFZP434I116 protein		
Hs.513041	0 (0.00) <	40 (0.46)	C15orf12	15q24	chromosome 15 open reading frame 12	GO:0006364	rRNA processing
Hs.489336	0 (0.00) <	36 (0.42)	SYAP1	Xp22.22	synapse associated protein 1, SAP47 homolog (Drosophila)		
Hs.438678	2 (0.03) <	32 (0.37)	TALDO1	11p15.5-p15.4	transaldolase 1	GO:0005975	carbohydrate metabolism
Hs.356123	0 (0.00) <	31 (0.36)	KRT8	12q13	keratin 8	GO:0007010	cytoskeleton organization and biogenesis
Hs.94672	0 (0.00) <	29 (0.34)	GCN5L1	12q13-q14	GCN5 general control of amino-acid synthesis 5-like 1 (yeast)	GO:0000004	biological process unknown
Hs.337307	0 (0.00) <	27 (0.31)	RPS27	1q21	ribosomal protein S27 (metallopanstimulin 1)	GO:0006412	protein biosynthesis
Hs.406515	0 (0.00) <	26 (0.30)	NQO1	16q22.1	NAD(P)H dehydrogenase, quinone 1	GO:0006809	nitric oxide biosynthesis
Hs.335918	3 (0.05) <	25 (0.29)	FDPS	1q22	farnesyl diphosphate synthase	GO:0006695	cholesterol biosynthesis
Hs.539	3 (0.05) <	24 (0.28)	RPS29	14q	ribosomal protein S29	GO:0006412	protein biosynthesis
Hs.74368	1 (0.02) <	23 (0.27)	CKAP4	12q24.11	cytoskeleton-associated protein 4	GO:0016021	integral to membrane
Hs.81256	0 (0.00) <	21 (0.24)	S100A4	1q21	S100 calcium binding protein A4		
Hs.201967	0 (0.00) <	19 (0.22)	AKR1C2	10p15-p14	aldo-keto reductase family 1, member C2	GO:0006629	lipid metabolism
Hs.23881	0 (0.00) <	19 (0.22)	KRT7	12q12-q13	keratin 7	GO:0007010	cytoskeleton organization and biogenesis
Hs.375921	2 (0.03) <	19 (0.22)	RPL31	2q12.1	ribosomal protein L31	GO:0006412	protein biosynthesis
Hs.177530	0 (0.00) <	18 (0.21)	ATP5E	20q13.3	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	GO:0015986	ATP synthesis coupled proton transport
Hs.347144	0 (0.00) <	18 (0.21)	dj55C23.6	6q22.3-q23.3	dj55C23.6 gene	GO:0006810	transport
Hs.433951	1 (0.02) <	16 (0.18)	GPX4	19p13.3	glutathione peroxidase 4 (phospholipid hydroperoxidase)	GO:0006644	phospholipid metabolism
Hs.5302	0 (0.00) <	13 (0.15)	LGALS4	19q13.2	lectin, galactoside-binding, soluble, 4 (galectin 4)	GO:0007155	cell adhesion
Hs.294584	0 (0.00) <	12 (0.14)	LTB4DH	9q32	leukotriene B4 12-hydroxydehydrogenase	GO:0006691	leukotriene metabolism
Hs.449070	19 (0.29) <	53 (0.61)	RPL13A	19q13.3	ribosomal protein L13a	GO:0006412	protein biosynthesis
Hs.406683	3 (0.05) <	20 (0.23)	RPS15	19p13.3	ribosomal protein S15	GO:0006412	protein biosynthesis
Hs.410900	0 (0.00) <	11 (0.13)	ID1	20q11	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	GO:0045449	regulation of transcription
Hs.436656	0 (0.00) <	11 (0.13)	SNRPG	2p13.3	small nuclear ribonucleoprotein polypeptide G	GO:0008380	RNA splicing
Hs.170622	8 (0.12) <	29 (0.34)	CFL1	11q13	cofilin 1 (non-muscle)	GO:0030036	actin cytoskeleton organization and biogenesis
Hs.77385	6 (0.09) <	25 (0.29)	MYL6	12q13.13	myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	GO:0007519	myogenesis
Hs.103833	0 (0.00) <	9 (0.10)	FLJ23469	19q13.42	hypothetical protein FLJ23469		
Hs.372960	0 (0.00) <	9 (0.10)	RPS21	20q13.3	ribosomal protein S21	GO:0006412	protein biosynthesis
Hs.436349	0 (0.00) <	9 (0.10)	RTN4	2p14-p13	reticulon 4	GO:0042981	regulation of apoptosis
Hs.386384	4 (0.06) <	19 (0.22)	RPS23	5q14.1	ribosomal protein S23	GO:0006412	protein biosynthesis
Hs.77060	6 (0.09) <	23 (0.27)	PSMB6	17p13	proteasome (prosome, macropain) subunit, beta type, 6	GO:0006511	ubiquitin-dependent protein catabolism
Hs.459927	7 (0.11) <	25 (0.29)	PTMA	2q35-q36	prothymosin, alpha (gene sequence 28)	GO:0006350	transcription
Hs.80545	2 (0.03) <	13 (0.15)	RPL37	5q13	ribosomal protein L37	GO:0006412	protein biosynthesis
Down-regulated in HBV-HCC cells							
Hs.446608	154 (2.34) >	104 (1.20)	K-ALPHA-1	12q13.12	tubulin, alpha, ubiquitous	GO:0051258	protein polymerization
Hs.2795	148 (2.25) >	47 (0.54)	LDHA	11p15.4	lactate dehydrogenase A	GO:0006096	glycolysis
Hs.435800	79 (1.20) >	5 (0.06)	VIM	10p13	vimentin		
Hs.256184	78 (1.18) >	59 (0.68)	EEF1G	11q12.3	eukaryotic translation elongation factor 1 gamma	GO:0006412	protein biosynthesis
Hs.129673	32 (0.49) >	12 (0.14)	EIF4A1	17p13	eukaryotic translation initiation factor 4A, isoform 1	GO:0006412	protein biosynthesis
Hs.283565	24 (0.36) >	0 (0.00)	FOSL1	11q13	FOS-like antigen 1	GO:0006968	cellular defense response
Hs.274485	22 (0.33) >	6 (0.07)	HLA-C	6p21.3	major histocompatibility complex, class I, C	GO:0019882	antigen presentation
Hs.287558	21 (0.32) >	3 (0.03)	ANXA1	9q12-q21.2	annexin A1	GO:0001501	skeletal development
Hs.57301	19 (0.29) >	5 (0.06)	HMGA1	6p21	high mobility group AT-hook 1	GO:0006345	loss of chromatin silencing
Hs.421257	19 (0.29) >	6 (0.07)	RPL7	8q13.3	ribosomal protein L7	GO:0006412	protein biosynthesis
Hs.181243	17 (0.26) >	5 (0.06)	ATF4	22q13.1	activating transcription factor 4 (tax-responsive enhancer element B67)	GO:0006355	regulation of transcription, DNA-dependent
Hs.418138	14 (0.21) >	3 (0.03)	FN1	2q34	fibronectin 1	GO:0007155	cell adhesion
Hs.436441	12 (0.18) >	1 (0.01)	LMNA	1q21.2-q21.3	lamin A/C	GO:0007517	muscle development
Hs.301853	8 (0.12) >	0 (0.00)	RAB34	17q11.2	RAB34, member RAS oncogene family	GO:0015031	protein transport
Hs.74034	13 (0.20) >	3 (0.03)	CAV1	7q31.1	caveolin 1, caveolae protein, 22kDa		
Hs.306278	13 (0.20) >	3 (0.03)	CD44	11p13	CD44 antigen (homing function and Indian blood group system)	GO:0016337	cell-cell adhesion
Hs.133892	9 (0.14) >	1 (0.01)	TPM1	15q22.1	tropomyosin 1 (alpha)	GO:0008016	regulation of heart contraction rate
Hs.157148	7 (0.11) >	0 (0.00)	MGC13204	12p13.33	hypothetical protein MGC13204		
Hs.398157	7 (0.11) >	0 (0.00)	SNK	5q12.1-q13.2	serum-inducible kinase	GO:0043123	positive regulation of I-kappaB kinase/NF-kappaB
Hs.426930	69 (1.05) >	54 (0.62)	ACTB	7p15-p12	actin, beta	GO:0006928	cell motility
Hs.169476	19 (0.29) >	8 (0.09)	GAPD	12p13	glyceraldehyde-3-phosphate dehydrogenase	GO:0006096	glycolysis
Hs.290404	12 (0.18) >	3 (0.03)	SLC25A3	12q23	solute carrier family 25, member 3	GO:0006091	generation of precursor metabolites and energy
Hs.73722	10 (0.15) >	2 (0.02)	APEX1	14q11.2-q12	APEX nuclease (multifunctional DNA repair enzyme) 1	GO:0006284	base-excision repair
Hs.368149	10 (0.15) >	2 (0.02)	CCT7	2p13.2	chaperonin containing TCP1, subunit 7 (eta)	GO:0000074	regulation of cell cycle
Hs.169744	13 (0.20) >	4 (0.05)	G22P1	22q13.2-q13.31	thyroid autoantigen 70kDa (Ku antigen)	GO:0006266	DNA ligation
Hs.145741	7 (0.11) >	1 (0.01)	ANXA5	4q26-q28	annexin A5	GO:0001501	skeletal development
Hs.79110	10 (0.15) >	3 (0.03)	NCL	2q12-qter	nucleolin	GO:0000398	nuclear mRNA splicing, via spliceosome
Hs.333786	10 (0.15) >	3 (0.03)	PSMA2	7p13	proteasome (prosome, macropain) subunit, alpha type, 2	GO:0006511	ubiquitin-dependent protein catabolism
Hs.425808	14 (0.21) >	6 (0.07)	CALM2	2p21	calmodulin 2 (phosphorylase kinase, delta)		
Hs.406620	21 (0.32) >	12 (0.14)	RPS10	6p21.31	ribosomal protein S10	GO:0006412	protein biosynthesis
Hs.77961	15 (0.23) >	7 (0.08)	HLA-B	6p21.3	major histocompatibility complex, class I, B	GO:0019882	antigen presentation

HCC cell lines: HCV: L5HLK1, L12J5HC, L16HLK3; HBV: L3SNU475, L3SNU354, L8SNU354, L11SNU354, L13SNU387

[a] Probability of differential expression calculated based on Audic and Claverie 1997. GO ids are based on process ontology of Gene Ontology database. Genes abundant (> 0.1%) in HCVor HBV are listed.

Colors indicate the frequency of a gene in each library.

0% 0.2 0.5 1 ≥ 5

identify genes related to the virological features of HCC. The examined HCC cell lines were divided into two groups: 'HBV pool,' composed of HBV-positive HCC cell lines; and 'HCV pool,' composed of HCC cell lines established from HCV-positive HCC tissues. From this analysis, we isolated 73 genes with frequencies of >0.1% on average from each pool and showing a significant difference (P>0.99) between the two pools, as shown in Table IV.

The genes in the HBV pool were found to contain 42 up-regulated genes (versus the HCV pool), which included genes such as *FTL*, *GNB2L1*, *GCN5L1*, *FDPS*, *S100A4* and *ID1*. Most of these genes have not been reported as being related to the development and carcinogenesis of HBV-associated HCC. A functional classification of these selected genes showed that genes involved in protein synthesis (*RPLP0*, *RPL5*, *RPS12*, *RPS27*, *RPS29*, *RPL31*, *RPL13A*, *RPS15*, *RPS21*, *RPS23*, and *RPL37*), cytoskeletal organization (*ANXA2*, *KRT8*, *CKAP4*, *KRT7*, and *CFL1*), xenobiotic and lipid metabolism (*AKR1C1*, *AKR1C3*, *NQO1*, and *AKR1C2*), metabolism (*ALDH1A1*, *TALDO1*, *GPX4*, and *LTB4DH*) and transport (*ATP5E* and *dj55C23.6*) were included. In addition, we observed 31 down-regulated genes in the HCV pool. In this case, significant differences were found for genes such as *VIM*, *FOSL1*, *HMGA1*, *ATF4* and *LMNA*. Many of these genes were involved in cell structure (*K-ALPHA-1* and *ACTB*), glycolysis (*LDHA* and *GAPD*), protein synthesis (*EEF1G*, *EIF4A1*, *RPL7*, and *RPS10*), antigen presentation (*HLA-C* and *HLA-B*), skeletal development (*ANXA1* and *ANXA5*), and cell adhesion (*FN1* and *CD44*) according to the GO term.

Verification of the genes related to HBV- or HCV-associated HCCs using semi-quantitative RT-PCR. To more quantitatively examine our data for hepatitis-virus infection in HCC, we randomly selected 18 up-regulated genes from the HBV pool and 12 down-regulated genes from the HBV pool. The expression levels of these selected genes were confirmed by semi-quantitative RT-PCR analysis in the cell lines. In a comparison between RT-PCR data obtained from the HBV pool and HCV pool, approximately 70% of the up-regulated and down-regulated genes were in agreement with the EST frequency data (data not shown). Fig. 3 represents the results

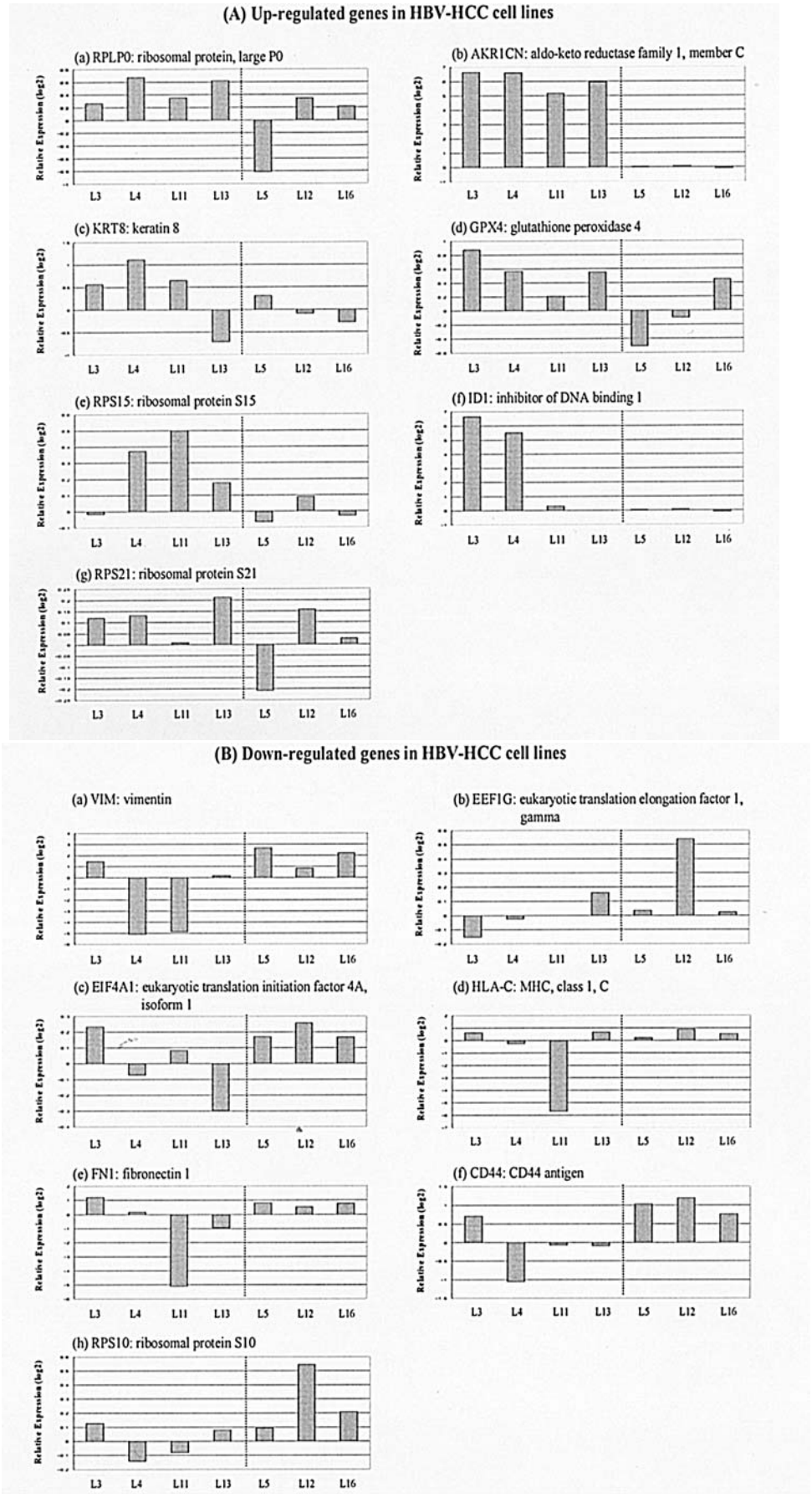


Figure 3. Semi-quantitative RT-PCR of the up- or down-regulated genes randomly selected from an HBV pool based on EST frequency. Total RNAs were extracted from HBV-HCC cell lines (L3, SNU475; L4, SNU368; L11, SNU354; L13, SNU387) and HCV-HCC lines (L5, HLK1; L12, JSHC; L16, HLK3). (A) Up-regulated and (B) down-regulated genes in the HBV pool. The transcript levels of the target genes were calculated using the same method described in Fig. 1, except that the transcript levels of target genes were calculated relative to the average amount of target gene in HCV pool (A) or relative to that of HBV cells (B). AKR1CN indicates the AKR1C family including AKR1C1, AKR1C2 and AKR1C3.

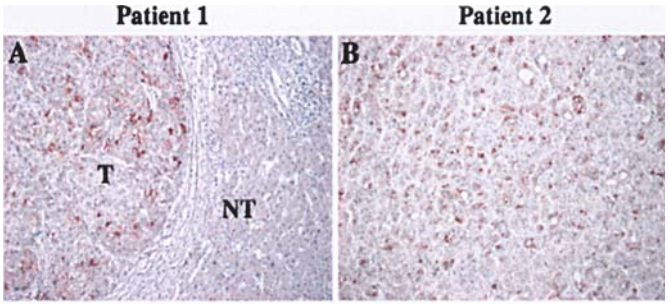


Figure 4. Immunohistochemistry of ANXA2 in HCC tissues. T represents the tumor region of HCC, and NT the non-tumor region. Original magnification, x100 (patient 1) and x200 (patient 2).

for the seven up-regulated genes and seven down-regulated genes that had a high expressional difference between the HBV pool and HCV pool. The genes selected as up-regulated genes in the HBV pool were highly expressed in most of the HBV pool (Fig. 3A), but expressed at low levels or not expressed in most of the HCV pool. On the other hand, most of the down-regulated genes were expressed at lower levels in the HBV pool compared to the HCV pool (Fig. 3B).

Verification of protein levels for selected genes using immunohistochemistry. To verify protein levels for the genes confirmed by semi-quantitative RT-PCR, an immunohistochemical analysis of 13 human liver cancer tissues, mainly comprised of well- to moderately differentiated grade I or II tumors, was performed. *ANXA2* and *ENO1* were selected as target genes for HCC, as antibodies were available and proteins previously confirmed to be related to HCC were excluded.

Among 13 human liver cancer tissues, the ANXA2 protein was shown to be highly expressed in the 10 HCC tumor tissues compared to their corresponding non-tumor tissues. As shown in Fig. 4, it was highly expressed in the HCC region (T) compared with the non-tumor liver region (NT), and distributed mainly in the cytosol and membrane regions. On the other hand, ENO1 proteins were not detected in either normal liver or HCC tumor tissues in our study. This is thought to be due to the low reactivity of antibodies against ENO1. These results indicate that an increase in mRNA levels for target genes such as ANXA2 in HCC are coupled to the protein levels. In addition, these results suggest that the selected target genes may be possible candidate markers for the identification of HCC.

Discussion

A global gene expression analysis of human cancer may provide important clues in understanding liver cancer oncogenesis and lead to improvements in predicting its clinical behavior. Using an analysis of EST frequency, we examined the difference in gene expression profiles between normal liver tissues and HCC cells, as well as between HBV-positive and HCV-associated HCC. Although we performed the gene expression analysis between normal liver tissues and HCC cell lines because normal liver cell lines were not available, data from these sets are consistent with RT-PCR data from HCC tissues and their corresponding non-tumor tissues. All HCC cell lines used in this study were obtained from Korean liver cancer patients. It has been reported that specific characteristics of genetic alterations in the original tumors are maintained during the establishment of immortalized cancer cells and, as a result, there is rarely a difference in genetic changes between the cancer cell line and original tumor (27,28). Our results are consistent with these reports in that the EST frequency data from HCC cell lines was in general agreement with RT-PCR data for the HCC cell lines and tissues. Among the selected genes, at least 28 genes and well-known HCC-related genes, as confirmed by semi-quantitative RT-PCR, could be identified as novel genes related to HCC, although some belong to the same functional category as genes previously reported to be related to liver cancer. These expression profiles may be useful for elucidating the molecular carcinogenesis of HCC, especially HBV- and HCV-associated HCC.

The gene expression profile between normal liver tissues and HCC cells showed that genes involved in the glycolytic pathway such as *LDHA*, *ENO1* and *PKM2* were increased in HCC, which is in agreement with other studies that reported an increase in glycolysis in many types of cancer (33,34). These glycolytic enzymes are known to be a hypoxia-inducible factor in other forms of cancer (35). This result indicates that the HIF-1 α signaling pathway might be related to the pathogenesis and progression of HCC. Several genes involved in protein translation were also highly expressed in HCC. Although most are known in HCC and in other cancers (36-38), *RPL4*, *RPL10* and *RPL13*, which were expressed at high levels in human HCC tissues with a frequency of >70%, have not been reported to be related to liver cancer, including *LDHA* and *ENO1* of the glycolytic pathway. The up-regulation of these genes was shown to be higher in grade III/IV carcinoma compared with those in grade I/II of HCC. Generally, HCC appears to develop as well-differentiated tumors (Edmondson grade I), then progress to moderately or poorly differentiated states (Edmondson grade II, III, or IV). In this respect, the expression of these genes was preferentially increased in moderately to poorly differentiated tumors, implying that an increased rate of protein synthesis is a major characteristic of tumor progression. In addition, heat shock proteins were found to be preferentially and highly expressed in HCC. It has been reported that HSP90 was identified as an essential host factor for hepatitis B virus replication (39) and HSP70 as a molecular marker for early HCC (40). These heat shock proteins are also known to be up-regulated in many cancers (41). Since components related to survival and apoptotic pathways are regulated by interactions with molecular chaperones such as the HSP70 and HSP90 family (41), these heat shock proteins may be a useful target region for the molecular prognosis and treatment of liver cancer. On the other hand, several genes for proteins associated with the metabolism of alcohol, drugs, glucose, lipids, and amino acids and in respiratory chain complexes were down-regulated in liver cancer. In addition, the levels of a number of liver-specific proteins such as albumin, transferrin, coagulation factor, and complement components were decreased, as has been reported previously (20). Among the down-regulated genes, *AMBP*, *GC, SERPINC1* and *A1BG* might be also

candidates for the diagnosis and treatment of liver cancer because they are known to be immune-related proteins or plasma proteins.

We also attempted to establish links between gene expression and the viral status of HCC. Comparative analysis of HBV- and HCV-associated HCC cell lines revealed that many ribosomal-related genes such as RPL family genes were up-regulated in HBV-associated HCC cell lines (HBV-HCCs) in comparison to HCV-associated HCC cell lines (HCV-HCCs), suggesting the activation of protein translation in HBV-HCCs. This observation is consistent with the previous report that major classes of genes encoding ribosomal proteins were up-regulated by the HBX protein (19). In addition, genes involved in extracellular matrix and cytoskeletal organization, such as *ANXA2*, *KRT8*, *CKAP4*, *KRT7* and *CFL1*, were shown to be up-regulated in HBV-HCCs, as well as genes such as *K-ALPHA-1*, *ANXA1*, *ACTB*, and *ANXA5* in HCV-HCCs. Our results support the hypothesis that the deregulation of genes encoding proteins associated with the cytoskeleton play a role in liver carcinogenesis (42). These findings also indicate that the pathway for liver carcinogenesis in the cytoskeleton may be different between HBV-HCCs and HCV-HCCs. A large number of genes implicated in the metabolism of xenobiotics, lipids, carbohydrates, nitric oxide and leukotriene were also overexpressed in HBV-HCCs. Most of these genes encode for key enzymes for chemotherapeutic drugs or xenobiotic carcinogens. In particular, AKR1C1, AKR1C2 and AKR1C3, which are members of AKR1C (aldo-keto reductase family 1, member C), were highly overexpressed in HBV-HCCs. These enzymes catalyze the conversion of aldehydes and ketones to the corresponding alcohols utilizing NADH and/or NADPH as cofactors. Enhanced expression of these enzymes suggests that a greater contribution of carcinogenic metabolites may be involved in the mechanism of HBV-specific hepatocarcinogenesis. These findings are consistent with previous reports on the overexpression of *AKR1D1* in HBV-HCC in comparison to HCV-HCCs (43), but not in complete agreement with previously reported findings such as overexpression of *AKR1D1* (44) and *AKR1C4* (17) in HCV-HCC. On the other hand, genes of HLA-B and HLA-C were highly expressed in HCV-HCCs. Our results are consistent with previous observations of increased expression of *HLA-B* and *HLA-C* in human hepatoma cell lines that constitutively express the HCV open reading frame (45) and increased expression of *HLA-B* in liver fibrosis associated with chronic HCV infection (46). In addition, cell adhesion genes such as *FN1* and *CD44* were found to be up-regulated in HCV-HCCs, but have not been reported to be related to human HCV-HCCs. Most obtained genes, which are expressed differently in the HBV HCCs vis-a-vis HCV-HCCs, were newly identified, although the up-regulation of genes such as *tubulin* and *HMGA1* in the HCV pool was in agreement with previous data (47). It has been suggested that liver carcinogenesis induced by HBV and HCV, in addition to common genetic and epigenetic alterations, may involve distinct pathways (47). Our expression profiles suggest that hepatitis viruses affect the expression of dozens of genes in HCC in a type-specific manner, thus invoking slightly different mechanisms of carcinogenesis. The identification of genes defining virus type-specific expression profiles would contribute to our ability to develop virus type-dependent treatment regimens.

Annexins are calcium-dependent phospholipid-binding proteins that play a role in the regulation of cellular growth and in signal transduction pathways (48). They are highly variable in their N-terminal region, which is known to mediate the specific functions of individual annexins. Our results indicate that transcripts of *ANXA2* are overexpressed in HCC, especially in HBV-HCCs compared to the HCV-HCCs, although *ANXA1* and *ANXA5* are slightly overexpressed in HCV-HCCs (data not shown). *ANXA2* has been reported to be increased in colorectal cancer (49), but decreased in prostate cancer (50). These reports indicate that these proteins have different functions, such as in tumor progression or tumor suppression, depending on the cancer type or specific stage. The expression of *ANXA2* in liver cancer has not yet been reported. Using immunohistochemistry, overexpression of the ANXA2 protein was also confirmed in HCC tissues. These results indicate that an increase in the mRNA level of *ANXA2* in HCC tissues coupled with that of the protein level and change in the amount produced are associated with carcinogenesis in HCC. From these results, *ANXA2* could be used as a marker for HCC.

We collected a total of approximately 21,000 unique ESTs (data not shown), and comprehensive cDNA microarray analyses using these clinical samples from several types of liver cancers are underway. In the gene expression profiling, our analysis using EST frequency has the advantage of permitting candidate genes to be selected without bias in comparison with the microarray analysis for only spotted genes. With the data obtained from EST frequency, further studies should provide a valuable resource for our understanding of liver cancer by revealing specific patterns that may reflect the prognosis and drug sensitivity of tumor cells, and identifying genes that are involved in the malignant transformation, progression, and metastasis of tumors. By raising antibodies against the products of these genes, we hope to identify new serum markers for the detection and diagnosis of HCC, and perhaps new candidate targets for treatment.

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